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Antigenic Properties and Diagnostic Potential of Recombinant Dobrava Virus Nucleocapsid Protein

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Dobrava hantavirus (DOBV) causes severe hemorrhagic fever with renal syndrome in the Balkan region and has been detected recently also in Russia, Estonia, and Germany. DOBV nucleocapsid protein (N) was produced in insect cells, using the baculovirus expression system (bac-DOBV-N), and in *E. coli* as a truncated (aa 1–165) glutathione-S transferase fusion protein (DOBV-dN-GST). The antigenic properties of bac-DOBV-N were found identical to native DOBV-N when examined by a panel of hantavirus-specific monoclonal antibodies. Enzyme immunoassays for detection of IgM and IgG antibodies were set up using DOBV recombinant N proteins and compared with those based on recombinant Hantaan and Puumala virus N, using panels of sera collected from DOBV, Hantaan and Puumala virus-infected patients. Full-length N protein (bac-DOBV-N) was found to be a more sensitive antigen than DOBV-dN-GST. The sensitivity values for sera from DOBV-infected patients were 100% for bac-DOBV-N and 86% for DOBV-dN-GST by IgM assays, and 98% for bac-DOBV-N and 88% for DOBV-dN-GST by IgG assays. The specificity values were 100% for bac-DOBV-N and 99% for DOBV-dN-GST by IgM assays, and 100% for both antigens by IgG assays. ***J. Med. Virol. 61:266–274, 2000.***

KEY WORDS: Dobrava hantavirus; hemorrhagic fever with renal syndrome; recombinant antigen; nucleocapsid protein; EIA; serology

INTRODUCTION

Dobrava virus (DOBV), a member of the Hantavirus genus in the family Bunyaviridae, is a causative agent of hemorrhagic fever with renal syndrome (HFRS) [Antoniadis et al., 1996; Avsic-Zupanc et al., 1992; Lundkvist et al., 1997]. Hantaviruses are negative-stranded RNA-viruses with a tripartite genome; the S segment encodes a nucleocapsid protein (N), the M segment two glycoproteins G1 and G2, and the L segment an RNA polymerase. Hantaviruses are transmitted to humans from rodent hosts, probably through inhalation of aerosolized excreta. The yellow-necked field mouse (*Apodemus flavicollis*) and the striped field mouse (*A. agrarius*) have been shown to carry DOBV in Europe [Avsic-Zupanc et al., 1992; Lundkvist et al., 1997b; Plyusnin et al., 1997]. Human infections have been reported from Albania, Greece, Bosnia-Herzegovina, Slovenia, Germany, Estonia, and Russia [Antoniadis et al., 1996; Avsic-Zupanc et al., 1999; Lundkvist et al., 1997a,b, 1998; Miesel et al., 1999]. The severity of HFRS caused by DOBV resembles that caused by Hantaan virus (HTNV), which circulates in Asia, as an overall mortality rate of 6.6% has been reported in the Balkans [Avsic-Zupanc et al., 1999]. However, in Russia and Estonia, no mortality has yet been associated with DOBV infections [Lundkvist et al., 1997a, 1998]. Puumala virus (PUUV), carried by the bank vole (*Clethrionomys glareolus*) is a more frequent cause of HFRS in most of Europe. HFRS caused by PUUV is similar to DOBV infections but is generally milder and is rarely manifested by hemorrhages. As DOBV and HTNV are serologically closely related, DOBV infections have been recorded previously to be caused by HTNV or Seoul virus (SEOV). Recent data [Antoniadis et al., 1996; Lundkvist et al., 1997a,b, 1998; Papa et al., 1998; Avsic-Zupanc et al., 1999] suggest, however, that only two pathogenic hantaviruses circulate in Europe: DOBV and PUUV.

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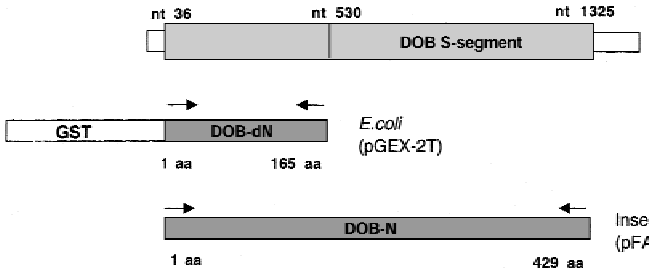

Fig. 1. Recombinant DOBV-N expression constructs.

The increasing awareness of human DOBV cases in Europe has created an urgent need for rapid and reliable diagnostic assays. As a highly pathogenic virus requiring Bio-safety level 3 facilities, other ways for production of viral antigen than growing in cell cultures are preferred. It is in the interest of clinical laboratories to know which of the hantavirus antigens should be used to diagnose infections in certain geographical regions.

Previously, hantavirus antibody enzyme immunoassays (EIAs) based on full-length N or truncated constructs expressed in *Escherichia coli* or insect cells have been developed [Kallio-Kokko et al., 1993, 1998; Elgh et al., 1996, 1997; Gött et al., 1997]. For the diagnosis of PUUV infection, assays based on the total N protein expressed in insect cells were found indistinguishable from assays based on native PUUV antigen [Vapalahti et al., 1996; Brus-Sjölander et al., 1997; Kallio-Kokko et al., 1998].

The aim of the present study was to produce recombinant DOBV nucleocapsid protein (DOBV-N) antigens in *E. coli* and in insect cells, and to evaluate the suitability of these recombinant proteins as diagnostic antigens using serum panels from various parts of Europe and from Korea. The possible differences between the use of whole and truncated DOBV-N were evaluated, as compared with native antigens as well as with recombinant HTNV and PUUV N proteins.

MATERIALS AND METHODS

Cloning and Expression of DOBV-N Protein in Insect Cells

Polymerase chain reaction (PCR) of the DOBV-N (nt 36–1325) for cloning into pFASTBAC1 vector (Bac-to-Bac™ Baculovirus Expression System, Gibco-BRL, Paisley, UK) was carried out by DOBV-specific primers containing *Bgl*II and *Xba*I restriction sites (initiation and stop codons and restriction sites underlined) (Fig. 1):

5′ primer: TTAGATCTCTATACTGAAGATGGCAACAC
3′ primer: TTTTCTAGAATACCTATTTAAAGC

The cDNA clone of the DOBV Saaremaa isolate S segment [Nemirov et al., 1999] was used as template for PCR, which was carried out using 50 pmol of both primers and 5 ng of template, following a standard PCR protocol: 30 cycles of 95°C, 40°C, 72°, using AmpliTaq-polymerase (Perkin-Elmer, NJ). The 1290-nt PCR segment was cut using *Bgl*II and *Xba*I, and the vector with *Bam*HI and *Xba*I, and run in 1.5% SeaPlaque agarose gel electrophoresis. The correct-size bands were cut off under ultraviolet (UV) light and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Ligation was carried out overnight at 16°C.

Recombinant baculovirus was constructed by transposition of the transfer vector to baculovirus DNA and transfection of the resulting recombinant bacmid DNA into Sf9 cells according to the manufacturer's instructions (Gibco-BRL). For production of antigen, Sf9 cells were infected for 72 hr at 25°C with the recombinant baculovirus, after which cells were collected by centrifugation. Proteinase inhibitors, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin (Boehringer Mannheim, Mannheim, Germany) and 1 mM EDTA, were added, and the cell extract was either stored at −70°C for future use, or immediately treated with urea to solubilize the 48-kDa N protein as described earlier [Vapalahti et al., 1996]. Fractions were run in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose filter, and immunoblotted using rabbit anti-DOBV serum [Lundkvist et al., 1997b] to test the antigenicity of the protein.

Cloning and expression of DOBV-N protein in *E. coli*. The PCR of the DOBV-dN (nt 36–530) for cloning into pGEX-2T vector (Pharmacia Biotech, Sollentuna, Sweden) was done using DOBV-specific primers containing *Bam*HI restriction sites (initiation and stop codons and restriction sites underlined) (Fig. 1).

5′ primer: TTTGGATCCAAGATGGCAACACTAGAGGAAC
3′ primer: TTTGGATCC
TCAAAAAGAACTGTCATCCTTAAATCG.

The cDNA of the DOBV Saaremaa isolate S segment [Nemirov et al., 1999] was used as a template for PCR, which was carried out using 50 pmol of both primers and 5 ng of template, following a standard PCR protocol 30 cycles of 95°C, 56°C, 72°, using AmpliTaq-polymerase (Perkin-Elmer, NJ). The 495-nt PCR segment as well as the pGEX-2T vector were cut using *Bam*HI, after which the vector was treated with calf intestinal phosphatase (New England BioLabs, Beverly, MA). The PCR fragment was purified, and ligation was performed as above. The plasmids were transformed into competent *E. coli* DH5α, using the heat-

shock method [Hanahan, 1983]. Plasmids were purified and restricted to confirm the right orientation of the insert. The linker regions were also sequenced to ensure the correct reading frame.

The bacteria were grown at +37°C, and the production of the fusion protein (glutathione-S-transferase (GST) plus aa 1–165 of DOBV-N) was induced by 1 mM IPTG (β-D-isopropylthiogalactopyranoside, Promega, Madison, WI). The cells were collected by centrifugation and treated with 1.5% sarkosyl (N-laurylsarcosine) to solubilize the otherwise aggregated fusion protein for glutathione-Sepharose bead purification as described earlier [Frangioni and Neel, 1993]. After the GST-fusion protein was attached into the beads, the protein was further purified using 10% SDS-PAGE. The correct-size band (44 kDa) was cut out, and the purified protein was recovered by electroelution (100 V, 4 hr) in 100 mM glycine-20 mM Tris-0.01% SDS. The antigenicity of the purified fusion protein was examined by immunoblotting, using rabbit anti-DOBV serum as described for bac-DOBV-N.

Other Recombinant Proteins

A recombinant baculovirus expressing HTNV-N [Schmaljohn et al., 1988] was kindly provided by Dr. Connie Schmaljohn, United States Army Medical Research Institute of Infectious Diseases (USAMRIID). The 48-kDa HTNV-N was solubilized using urea treatment as described earlier [Vapalahti et al., 1996]. Cloning and production of bac-PUUV-N (50 kDa) antigen have been described earlier [Vapalahti et al., 1996]. Cloning of PUUV-1b-GST (aa 1–118 of N) has been described earlier [Lundkvist et al., 1996a]. The 40-kDa PUUV-1b-GST was expressed and purified as described above to obtain pure soluble antigen.

Serum Panels

A total of 60 DOBV-positive sera from 28 patients were tested (including 11 patients with single sera, and 17 patients with 49 paired or serial sera): eight single sera from healthy blood donors from Estonia [Lundkvist et al., 1998], 13 single or paired sera from 8 HFRS patients from Bosnia [Lundkvist et al., 1997b], and 39 serial sera from 12 HFRS patients from Slovenia. All patients were confirmed to be DOBV-specific, using focus reduction neutralization test (FRNT) [Lundkvist et al., 1997b] Of 60 DOBV-positive sera, 28 were acute-phase samples (taken within 1 month after onset of symptoms).

Twenty HTNV-positive single acute-phase sera, kindly provided by Dr. Ho Wang Lee (Seoul, Korea) were tested. Twenty-three paired or serial PUUV-positive sera from 10 patients were obtained from our routine laboratory (HUCH Diagnostics, Helsinki). These sera were verified to be PUUV positive, using a PUUV-IgG immunofluorescence assay (IFA) [Hedman et al., 1991; Vapalahti et al., 1995]. Of the 23 sera, 18 were acute-phase samples, as determined by IgG-avidity assay [Hedman et al., 1991].

All positive serum panels were examined by RapiTex®RF (Behring, Marburg, Germany) [Schmitz and Folds, 1993] according to the manufacturer's instructions to evaluate whether they contained any rheumatoid factor (RF) that could interfere in direct IgM assays.

A panel of 40 PUUV- and DOBV-negative sera (negative by PUUV- and DOBV-IgG IFA) obtained from our routine diagnostic laboratory (sera had been sent for determination of PUUV antibodies) was used to evaluate the cut-off levels of the EIAs. Sera were chosen so that they had been collected later than the 6th day after onset of acute disease.

A panel of 50 additional hantavirus negative sera from Bosnia were tested. The sera were verified hantavirus negative, using native PUUV, HTNV, and DOBV based IgG- and IgM-EIAs [Brus-Sjölander et al., 1997].

Buffers and Serum Dilutions

In this investigation, 50 mM carbonate buffer, pH 9.6, was used for antibody-coating in all capture assays, and phosphate-buffered saline (PBS), pH 7.0, for antigen coating in all direct assays. The postcoating (blocking) step was performed by incubation with 3% bovine serum albumin (BSA)-PBS for 1 hr at +37°C. All sera, conjugates, as well as the antigens used in the following steps of the EIAs were diluted in PBS-0.05% Tween (PBST)-0.5% BSA. Serum samples were diluted 1:200 in all assays. The plates were washed five times (PBST) between each step. Specific binding of antibodies was determined using 3,3′5,5′-Tetramethylbenzidine as substrate (Sigma, St. Louis, MO) for 15 min at room temperature, after which the reaction was stopped with 2 M H_2SO_4, and the plates read at 450 nm.

Monoclonal Antibody Detection Assay

A panel of hantavirus-specific monoclonal antibodies (MAbs) was used to compare the antigenic properties of the recombinant proteins with the corresponding native antigens. The panel contained PUUV-N specific MAbs, 1C12, 4C3, 3E11, 3G5, and 2E12 [Lundkvist et al., 1991]; the PUUV-G2 specific MAb, 5B7 [Lundkvist and Niklasson, 1992]; HTNV-N specific MAbs, G6, F23A1, E5, and C16D11 [Yoshimatsu et al., 1996]; and the Tula hantavirus N specific MAb, 1C8 [Lundkvist et al., 1996b]. The antigenic recognition sites of the MAbs are shown in Table I. The plates were coated with bac-DOBV-N (1:100), DOBV-dN-GST (1:1,000), bac-HTNV-N (1:1,000), or bac-PUUV-N (1:800) overnight at room temperature, after which they were blocked before use. The MAbs, and positive and negative control patient sera were incubated in duplicate for 1 hr at +37°C. Specific antibody binding was detected by incubation of peroxidase-conjugated anti-mouse-Ig (DAKO, Clostrup, Denmark) diluted 1:2,000, or peroxidase-conjugated anti-human IgG (Cappel, Turnhout, Belgium) diluted 1:20,000, for 1 hr at +37°C.

TABLE I. MAb Reactivity of the Recombinant Proteins, Measured Using EIA*

MAb	Virus antigen, MAb recognition site (epitope specificity)	Recombinant antigen (aa)				Native antigen	
		bac-DOBV-N (aa 1–429)	DOBV-dN-GST (aa 1–165)	bac-HTNV-N (aa 1–429)	bac-PUUV-N (aa 1–433)	Native DOBV IFA	Native PUUV IFA
1C12	PUUV-N, aa 1–79 (N-f)	+	+	+	+	+	+
4C3	PUUV-N, aa 1–79 (N-h)	+	+	+	+	+	+
3 E 11	PUUV-N, aa 1–79 (N-f)	+	+	+	+	+	+
3G5	PUUV-N, aa 1–79 (N-d)	–	–	–	+	–	+
2 E 12	PUUV-N, aa 1–79 (N-g)	+	+	+	+	+w	+
5B7	PUUV-G2 (G2-b)	–	–	–	–	–	+
G6	HTNV-N, aa 166–176	+	–	+	+	+	+
F23A1	HTNV-N, aa 205–402	+	–	+	+	+	+
E 5	HTNV-N, aa 166–175	+	–	+	+	+	+
C16D11	HTNV-N, aa 244–429	+	–	+	+	+	+
1C8	TULV-N, aa 1–79 (N-e)	–	–	–	+	–	+

*MAb, monoclonal antibody; EIA, enzyme immunoassay; aa, amino acids; w, weak reaction.

μ-Capture IgM Assays

Plates were coated with anti-human IgM (Cappel, Turnhout, Belgium) diluted 1:500 overnight at room temperature, after which they were blocked before use. Serum samples were incubated in duplicate for 1 hr at +37°C, followed by the recombinant antigen (bac-DOBV-N diluted 1:25, bac-PUUV-N diluted 1:4,000, or bac-HTNV-N diluted 1:1,000) for 1 hr at +37°C, and the peroxidase-conjugated MAb 1C12 [Lundkvist et al., 1991] diluted 1:1000 for 1 hr at +37°C. Capture assay format was chosen for bac-DOBV-N and bac-HNTV-N because it was found to increase the sensitivity and to minimize unspecific reactions as compared with direct coating of the antigen (data not shown). For bac-PUUV-N the same has been shown earlier [Kallio-Kokko et al., 1998].

Direct IgM assay. For the DOBV-dN-GST IgM assay the plates were coated with DOBV-dN-GST (1:2000) overnight at room temperature, after which they were blocked before use. Serum samples in duplicate were incubated for 1 hr at +37°C, followed by peroxidase-conjugated anti-human IgM (DAKO, Clostrup, Denmark) diluted 1:1,000, for 1 hr at +37°C. Direct coating was chosen for DOBV-dN-GST because it was found to be more sensitive than capture assay (data not shown) possibly due to the size of the recombinant protein.

Antigen-Capture IgG Assays

Plates were coated with 1 μg/ml MAb 1C12 [Lundkvist et al., 1991] overnight at room temperature, after which they were blocked before use. Bac-DOBV-N antigen (1:400) or bac-HTNV-N (1:1,000) was added and incubated for 1 hr at +37°C (dilution buffer was used as negative control antigen for each serum sample). Sera in duplicate were incubated for 1 hr at +37°C, followed by peroxidase-conjugated anti-human IgG (Cappel, Turnhout, Belgium) diluted 1:20,000 for 1 hr at +37°C. Capture assay was chosen for these antigens because it was found to increase the sensitivity and to minimize unspecific reactions as compared with direct coating of the antigens (data not shown).

Direct IgG Assays

Plates were coated with DOBV-dN-GST (1:2,000), bac-PUUV-N (1:1,000), or PUUV-1b-GST (1:1,600) overnight at room temperature, after which they were blocked. Sera were incubated for 1 hr at +37°C in duplicate, followed by peroxidase-conjugated anti-human IgG (Cappel, Turnhout, Belgium) diluted 1:20,000 for 1 hr at +37°C. Direct coating was chosen for DOBV-dN-GST and PUUV-1b-GST because it was found to be more sensitive than capture assay (data not shown) possibly due to the sizes of the recombinant proteins. For bac-PUUV-N, the direct coating was found earlier to be more sensitive than the capture assay, especially in the acute phase [Kallio-Kokko et al., 1998].

Cut-off and Gray-Zone Values

For all assays, the cut-off values were calculated as the mean absorbance value of 40 negative controls plus three times the standard deviation. The positive sera (absorbance value > cut-off value) were divided into (1) gray-zone sera, and (2) high positive sera (absorbance value over gray-zone value), to demonstrate possible differences between the affinities of different assays. The gray-zone values (i.e., very low positives) were chosen as the absorbance values between the cut-off value and the cut-off value plus 0.2 absorbance units.

RESULTS

Expression of the Recombinant Proteins

Bac-DOBV-N produced in insect cells containing the full-length N, and DOBV-dN-GST produced in *E. coli* and containing GST and aa 1–165 of N, were both detected with a polyclonal rabbit anti-DOBV serum (Fig. 2), and were shown to be of expected sizes (48 kDa and 44 kDa, respectively). The expression level of DOBV-dN-GST was high (Fig. 2) with a yield of approximately 0.5–1 mg protein out of 200 ml *E. coli* culture, enough for coating of 50–100 EIA 96-well microtiter plates. The expression level of bac-DOBV-N was lower, the protein band being visually distinguishable only by immunoblotting (Fig. 2); 1 L of Sf9 cell suspension culture (2 ×

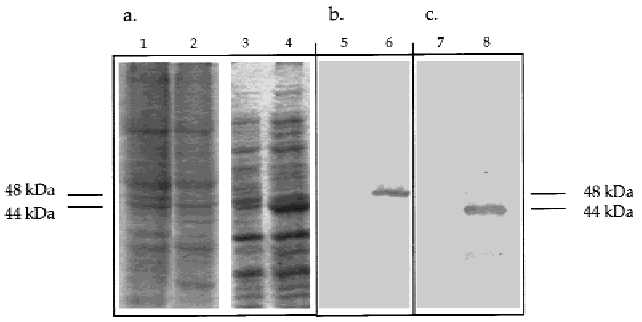

Fig 2. **a:** Coomassie blue staining of (1) Sf9 cell control, (2) Bac-DOBV-N (48 kDa) expressed in Sf9 cells, (3) *E. coli* control, and (4) DOBV-dN-GST (44 kDa) expressed in *Escherichia coli*. **b:** Immunoblotting of bac-DOBV-N (48 kDa) with (5) negative control serum and (6) rabbit anti-DOBV-serum. **c:** Immunoblotting of DOBV-dN-GST (44 kDa) with (7) negative control serum and (8) rabbit anti-DOBV serum.

10^6 cells/ml) yielded recombinant protein suitable as EIA antigen for 25–400 plates.

Antigenic Properties of the Recombinant Proteins

The hantavirus-specific MAbs reacted with bac-DOBV-N in EIA in a pattern similar to that of native DOBV antigen in IFA (Table I). DOBV-dN-GST lacks the epitopes located C-terminal from aa 165, and thus the HTNV-specific MAbs, (recognizing epitopes located in the region aa 166–429) did not react with the truncated DOBV-N. The PUUV- and TULV-specific MAbs (recognizing N-terminal epitopes aa 1–79) reacted with DOBV-dN-GST and bac-DOBV-N in a similar pattern (Table I).

IgM Assays

Altogether 60 DOBV-positive sera, 20 HTNV-positive sera, and 23 PUUV-positive sera were analyzed by IgM assays, using bac-DOBV-N, DOBV-dN-GST, bac-HTNV-N, and bac-PUUV-N antigens. Of the sera tested, 28 represented acute (sample taken within the first month after onset of illness) and 32 convalescent/old DOBV infection, 20 sera represented acute HTNV infection, and 18 sera acute and 5 sera convalescent/old PUUV infection. No false-positive reactions were found among the 90 negative control sera tested, when using bac-DOBV-N, bac-HTNV-N, and bac-PUUV-N antigens. One serum out of 90 negative control sera gave a false positive result using DOBV-dN-GST. The difference between bac-DOBV-N and DOBV-dN-GST was apparent when comparing the results of serial DOBV sera; in three patients out of 28 the diagnosis would have been completely missed by using only DOBV-dN-GST IgM assay (Fig. 3; patients marked by asterisk), whereas all acute DOBV sera were detected by bac-DOBV-N. All HTNV-sera reacted with bac-DOBV-N in IgM-assay, the absorbance values were, however, in the gray-zone level more often than with bac-HTNV-N. All DOBV-sera reacted also with bac-HTNV-N. The PUUV-positive sera showed low cross-reactivity to the DOBV- and HTNV-recombinant antigens and correspondingly, the DOBV- and HTNV-positive sera showed low cross-reactivity to bac-PUUV-N (Table II). None of the DOBV- or PUUV-positive old immunity sera (sample taken later than 3 months after onset of illness) showed reactivity in bac-DOBV-N, bac-HTNV-N or bac-PUUV-N IgM-assays, and only one was IgM-positive using DOBV-dN-assay (Table II). None of the sera tested was found to be RF-positive. The sensitivity values (calculated as the ratio of correct-positive EIA results of acute immunity sera to correct-positive results and false-negative EIA results) as well as the specificity values (calculated as the ratio of correct-negative EIA results to false-positive and correct-negative EIA results) for all IgM assays are shown in Table II. Because of the cross-reactivity between hantaviruses, the sensitivity values of each antigen were calculated individually for each hantavirus-positive serum panel, and the specificity values of each antigen using a hantavirus negative serum panel.

IgG Assays

Altogether 60 DOBV-positive sera (28 acute and 32 convalescent or old-immunity sera), 20 HTNV-positive sera, and 23 PUUV-positive sera were analyzed by IgG assays using bac-DOBV-N, DOBV-dN-GST, bac-HTNV-N, bac-PUUV-N, and PUUV-1b-GST antigens. No false-positive reactions were found among the 90 negative control sera tested, when using bac-DOBV-N, bac-HTNV-N, bac-PUUV-N, and DOBV-dN-GST antigens. One serum out of 90 negative control sera gave a false-positive result using PUUV-1b-GST. Bac-DOBV-N was again more sensitive than DOBV-dN-GST for detecting DOBV-specific antibodies (Table III). Bac-DOBV-N did not detect DOBV IgG antibodies in one early, IgM positive sample taken 5 days after onset of illness. Later samples of this patient (taken 14 days or later after onset of illness) were IgG positive. HTNV sera were detected with recombinant DOBV antigens less efficiently than with bac-HTNV-N (Table III). Bac-HTNV-N missed only one DOBV serum. The specificity and the sensitivity values of the assays (calculated as above) are shown in Table III.

DISCUSSION

Dobrava hantavirus was recently shown to be a distinct human pathogen circulating in central and eastern Europe either by reverse transcription (RT)-PCR and direct sequencing from patient samples [Antoniadis et al., 1996] or by focus-reduction neutralization tests (FRNT), including all hantaviruses known to cause HFRS [Avsic-Zupanc et al., 1999; Lundkvist et al., 1997a,b, 1998; Meisel et al., 1999; Plyusnin et al., 1997]. DOBV is closely related both genetically and serologically to HTNV, which circulates in Asia. The earlier false reports on HTNV and SEOV infections in Europe are examples of the difficulties in hantavirus typing based on serology using IFA, EIA or immunoblotting methods. It appears that the only reliable test for distinguishing the antibody responses against dif-

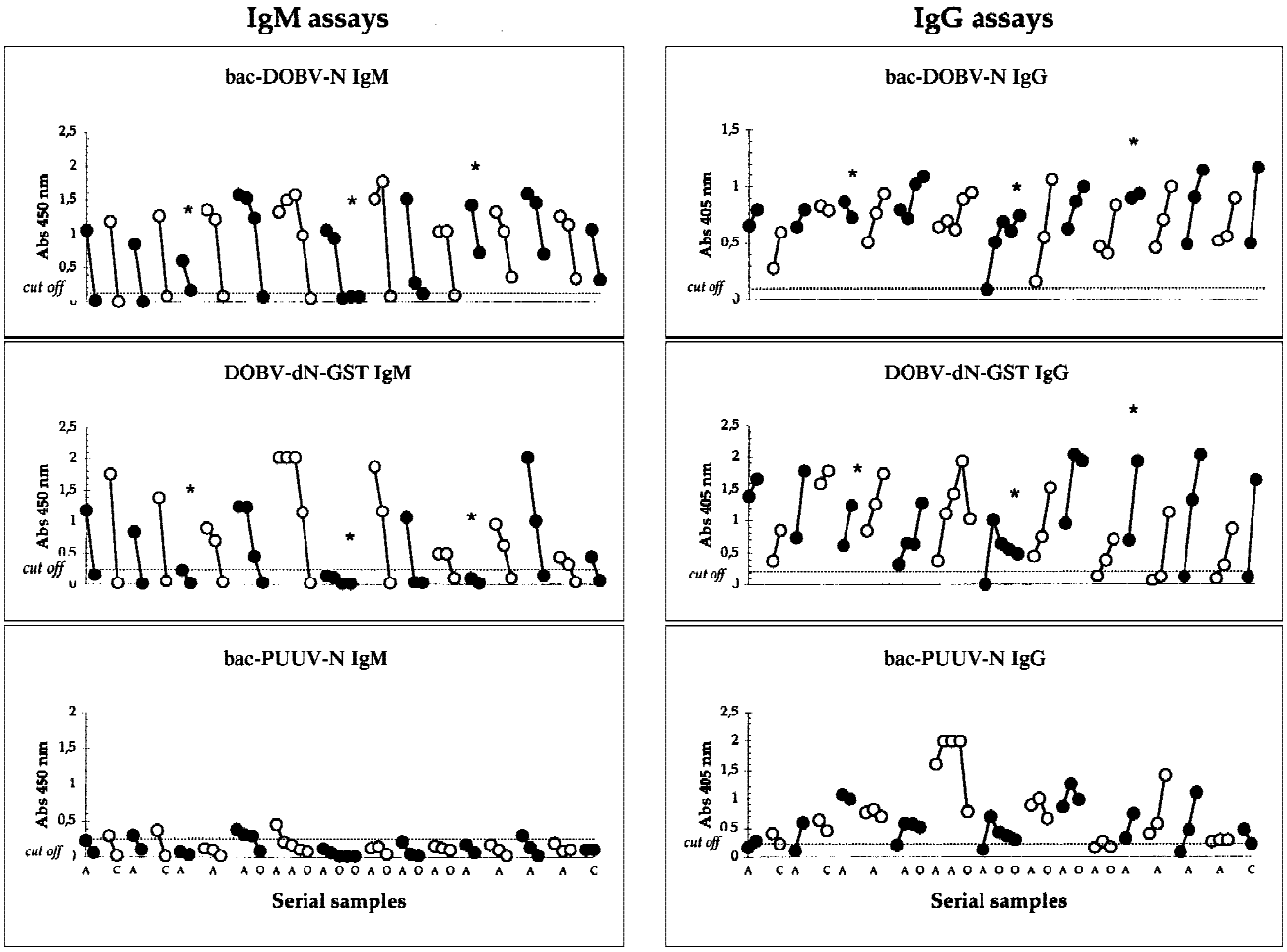

Fig 3. DOBV-positive serial sera (49 sera from 17 DOBV patients) tested in IgM and IgG assays, using bac-DOBV-N, DOBV-dN-GST, and bac-PUUV-N antigens. A, acute immunity sera, collected <1 month after onset of symptoms; C, convalescent sera, collected 1–3 months after onset of symptoms; O, old immunity sera, >3 months after onset of symptoms. *The patients whose diagnosis would have been missed if using only DOBV-dN-GST IgM-assay.

ferent closely related hantaviruses is the neutralization test (FRNT or plaque reduction neutralization test), with the maturation of antibodies also crucial for the typing [Lundkvist et al., 1997b]. For diagnostic purposes, however, it is most important that the tests detect early seroresponses with sensitivity against the local hantavirus(es), and distinction between closely related viruses, for example, cannot generally be achieved without loss of sensitivity [Morii et al., 1998].

Serological assays are needed for hantavirus diagnostics, as only about 67% of PUUV- patients [Plyusnin et al., 1999] and about 40% of DOBV patients [Papa et al., 1998] are virus-RNA positive by RT-PCR, and the isolation of hantaviruses from HFRS patients is rarely successful. For the serological diagnosis of hantavirus infections, the assays measuring IgM antibodies have been shown to be most informative, in hantavirus infections the IgM levels rise earlier as compared with the IgG antibodies, and the IgM production is clearly associated to acute infection [Brus-Sjölander et al., 1997; Kallio-Kokko et al., 1998]. The format of an assay can have a major impact on the specificity and sensitivity of the assay. If the expression level of an antigen is low, the use of capture assay may increase the sensitivity and specificity of the assay by minimizing the need for purification of the antigen. The length of the recombinant protein can affect its capacity for binding antibodies against different epitopes and may thus lower the sensitivity if capture assays are being used [Brus-Sjölander et al., 1997]. In this study, the most sensitive and specific assay for each antigen was chosen for analysis.

So far, mainly truncated recombinant antigens have been reported for use in assays for diagnosing DOBV infections in Europe, either recombinant DOBV-N (aa 1–117) [Elgh et al., 1997] or HTNV-N (aa 1–117 or aa 1–119) [Elgh et al., 1997; Gött et al., 1997]. Although our truncated construct (DOBV-dN-GST, aa 1–165) was longer than the constructs described previously, the full-length N protein was shown to be a more sensitive antigen: the IgM assay as well as the IgG assay based on the truncated antigen missed detection of DOBV antibodies in several sera (Fig. 3). Using a large panel of Mabs, the antigenic properties of bac-DOBV-N

TABLE II. Results of IgM EIAs Based on Different Hantavirus Antigens

	Negat. sera (tot n = 90)		DOBV-pos. sera (tot n = 60) (acute n = 28)		(conval./old n = 32)		HTNV-pos. sera (tot n = 20) (acute)		PUUV-pos. sera (tot n = 23) (acute n = 18)		(conval./old n = 5)	
Antigen	No pos.	Specif. (%)	No pos. (high/gray zone)	Sensit. (%)	No pos. (high/gray zone)	% posit.	No pos. (high/gray zone)	Sensit. (%)	No pos. (high/gray zone)	Sensit. (%)	No pos. (high/gray zone)	% posit.
bac-DOB-N	0	100	28 (28/0)	100	10[a] (7/3)	31	20 (14/6)	100	2 (0/2)	11	0	0
DOB-dN-GST	1	99	24 (22/2)	86	4[b] (3/1)	13	18 (17/1)	90	5 (2/3)	28	1[a] (1/0)	25
bac-HTN-N	0	100	28 (28/0)	100	8[a] (5/3)	25	20 (20/0)	100	5 (3/2)	28	0	0
bac-PUU-N	0	100	8 (1/7)	29	1[a] (0/1)	3	8 (3/5)	40	18 (16/2)	100	0	0

EIA, enzyme immunoassay.
[a]All positive sera are convalescent samples (taken 1–3 months after onset of symptoms).
[b]Three positive sera are convalescent samples, and one positive serum an old immunity sample (taken later than 3 months after onset of symptoms).

TABLE III. Results of IgG EIAs Based on Different Hantavirus Antigens

	Negat. sera (tot n = 90)		DOBV-pos. sera (tot n = 60)			HTNV-pos. sera (tot n = 20) (acute)		PUUV-pos. sera (tot n = 23)		
			(acute n = 28)	(conval./old n = 32)	Tot			(acute n = 18)	(conval./old n = 5)	Tot
Antigen	No pos.	Specif. (%)	No pos. (high/gray zone)	No pos. (high/gray zone)	Sensit. (%)	No pos. (high/gray zone)	Sensit. (%)	No pos. (high/gray zone)	No pos. (high/gray zone)	Sensit. (%)
bac-DOB-N	0	100	27 (26/1)	32 (32/0)	98	15 (14/1)	70	4 (1/3)	3 (1/2)	30
DOB-dN-GST	0	100	21 (19/2)	32 (32/0)	88	13 (8/5)	65	2 (0/2)	2 (0/2)	17
bac-HTN-N	0	100	27 (24/3)	32 (32/0)	98	20 (18/2)	100	7 (7/0)	4 (1/3)	48
bac-PUU-N	0	100	21 (16/5)	27 (18/9)	80	5 (1/4)	25	18 (18/0)	5 (5/0)	100
PUU-1b-GST	1	99	23 (12/11)	22 (11/11)	75	9 (3/6)	45	22 (21/1)	5 (5/0)	96

*EIA, enzyme immunoassay.

were identical to native DOBV-N, although the absence of DOBV-specific MAbs hindered a more complete antigenic evaluation.

One aim of this study was to evaluate the suitability of EIAs based on other hantavirus antigens (mainly HTNV and PUUV) for diagnosing DOBV cases in clinical laboratories in Europe. For clarity, the evaluation was to be carried out on a DOBV serum panel that had been fully confirmed by cross-FRNTs using several hantaviruses and also convalescent sera. In our study, bac-HTNV-N was found to have almost similar ability for detecting DOBV antibodies as bac-DOBV-N. This result is in line with the high serological cross-reactivity within the HTNV/DOBV/SEOV group and indicates that the full-length HTNV-N antigen is generally appropriate for detection of DOBV antibodies. However, recent data have shown that EIAs based on HTNV antigen fail in some cases to detect DOBV-specific antibodies, both in acute-phase and convalescent samples from HFRS patients [Brus-Sjölander and Lundkvist, 1999]. Bac-DOBV-N was not as good for the detection of HTNV-specific antibodies as bac-HTNV-N, which further supports the use of homologous antigens for reliable diagnosis of HFRS. The results on the comparison of bac-DOBV-N and bac-HTNV-N may, however, have been affected by the expression levels: the expression level of bac-DOBV-N was significantly lower as compared with our earlier experience with baculovirus-expressed HTNV-N and PUUV-N, possibly due to a different baculovirus expression system (Bac-to-Bac™) used. Despite the low expression level of bac-DOBV-N, the need of purification of the antigen could efficiently be circumvented by use of the capture format of the EIAs.

As PUUV and DOBV are to date the only known pathogenic hantaviruses in Europe, and both have been shown to circulate in large as well as overlapping regions in Europe, the need for diagnostic assays capable of detecting both antibodies is obvious. In conclusion, the baculovirus-expressed full-length nucleocapsid proteins are optimally sensitive and specific antigens for hantavirus serology. For diagnostic purposes in Europe, we recommend the use of full-length PUUV-N and DOBV-N antigen based EIAs to ensure accurate serology.

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